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Thermogenesis utilization of drip-dry injected iron from different iron sources in target tissues of broiler chickens

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Abstract
The information available regarding the utilization of iron (Fe) from different Fe sources at a target tissue level is not well known. It detect differences in Fe metabolic utilization among Fe sources, the effect of intravenously injected Fe on growth performance, hematological indices, tissue Fe concentrations and Fe-containing enzyme activities and gene expressions of Fe-containing enzymes or protein in broilers was investigated. On d 20 post-hatching, a total of 535 male chickens were randomly allotted to 1 of 9 treatments in a completely randomized design. Chickens were injected with either a 0.9% (wt/vol) NaCl solution (control) or a 0.9% NaCl solution supplemented with Fe sulphate or 1 of 3 organic Fe sources. The 3 organic Fe sources were Fe chelates with weak, moderate or extremely strong chelation strengths. The 2 Fe dosages were calculated according to the Fe absorbabilities of 10% and 20% every 2 d for a duration of 20 d. Iron injection did not affect (P > 0.05) ADFI, ADG or FCR during either 1 to 10 d or 11 to 20 d after injections. Hematocrit and Fe concentrations in the liver and kidney on d 10 after Fe injections, and Fe concentrations in the liver or pancreas and ferritin heavy-chain protein expression level in the liver or spleen on d 20 after Fe injections increased (P ≤ 0.05) as injected Fe dosages increased. When the injected Fe level was high at 20% Fe absorbability, the chickens injected with Fe-ProtES had lower (P < 0.001) kidney Fe concentrations and spleen FTH1 protein levels than those injected with Fe-MetW on d 20 after injections. And they had lower (P < 0.02) liver cytochrome c oxidase mRNA levels on d 20 after injections than those injected with Fe-Prot W or Fe sulphate. this study indicate that intravenously injected Fe from Fe-ProES was the least utilizable and functioned in the sensitive target tissue less effectively than Fe from Fe sulfate.

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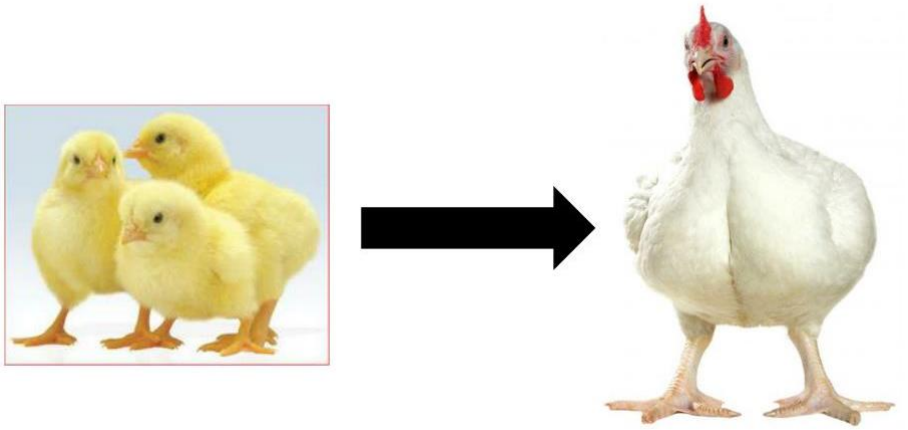
I. Introduction

Iron (Fe) is an essential trace element required in numerous important biological processes of animals. Rapidly growing chicks have a high demand for Fe, so Fe additives are routinely supplemented into diets for optimal growth. Traditionally, Fe is added to diets in the form of inorganic salts which have many disadvantages, such as low bioavailability, high hydroscopicity and oxidation. In recent years, organic Fe sources have been developed as alternatives to traditional inorganic Fe sources. However, reported results on bioavailabilities of organic Fe sources are inconsistent. Previous studies from our laboratory indicated that the bioavailabilities of organic Fe sources for broilers were closely correlated with their chelation strengths between Fe and their ligands. The Fe proteinate with moderate chelation strength is more available than iron sulfate in enhancing hemoglobin (Hb) and total body Hb Fe of broilers fed a casein–dextrose diet. Relative to Fe sulfate (assigned 100%), the bioavailabilities of organic Fe sources with weak, moderate and extremely strong chelation strength for broilers fed a conventional maize–soybean meal basal diet were 129%, 164% and 174%, respectively, therefore, organic Fe sources with greater Qf values showed higher Fe bioavailabilities. However, it is not clear whether the differences in bioavailabilities of Fe from different sources were due to the differences in Fe absorption or in Fe metabolic utilization, or in both aspects because the method of Fe administration in the above studies was dietary supplementation. Recent studies from our laboratory have further indicated that organic Fe sources with greater Qf values showed higher Fe absorption in the small intestine of broilers. However, different absorptions of organic Fe 69 sources in the small intestine of broilers could not fully explain the differences in their bioavailabilities, and thus, part of them might be associated with the different metabolic utilization of Fe from organic Fe sources at a target tissue level. However, no studies on this aspect have been reported before.



Direct injection of Fe sources into a vein might be an effective method to study the Fe metabolic utilization at a target tissue level by bypassing intestinal absorption. Previous studies from our laboratory demonstrated that an intravenously injected organic manganese (Mn) or zinc (Zn) source with strong chelation strength had the lowest Mn or Zn utilization in the target tissues of broilers. We hypothesized that the intravenously injected organic Fe source with extremely strong chelation strength would have the lowest Fe utilization in the target tissues of broilers. Therefore, the objective of the present study was to investigate the effect of intravenously injected Fe from different Fe sources on growth performance, hematological parameters, tissue Fe concentrations and Fe-containing enzyme activities and gene expressions of Fe-containing enzymes or protein to detect the differences in metabolic utilization of Fe from different Fe sources in the target tissues of broilers.

II. Process

Animal ethics

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the ARRIVE guidelines for reporting animal research. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

Experimental design and treatments

A completely randomized design was adopted in this experiment. The 9 treatments included a 0.9% (wt/vol) NaCl injection solution without Fe (the control), and the 0.9% saline solution supplemented with either Fe sulphate ($FeSO_4 \cdot 7H_2O$, reagent grade; 19.5% Fe by analysis**)** or 1 of 3 organic Fe sources, at 2 injected Fe dosages. The 3 organic Fe sources used in the current study were the same as those used in our previous studies, and they included Fe methionine with weak chelation strength (Fe-MetW, feed grade; 14.7% Fe and Qf = 1.37 by analysis), Fe proteinate with moderate chelation strength (Fe-ProtM, feed grade;14.2 % Fe and Qf = 43.6 by analysis), and Fe proteinate with extremely strong chelation strength (Fe-ProtES, feed grade;10.2% Fe and Qf = 8,590 by analysis). It was assumed that the amount of Fe injected should be close to the normal amount of Fe absorbed when chickens were fed a diet containing the optimal Fe. Therefore, the injected dosage of Fe was calculated using the following equation: Fe injected (mg/bird) = Fe absorbability (%) × Average daily feed intake (kg/d) × Dietary supplemental Fe level (40 mg/kg) × 2 (d). It has been reported that dietary Fe absorption in animals ranges from about 5% to 30%, so the values of 10% and 20% were used. The average daily feed intake was adjusted every 7 d based on the feed intakes from 22 to 42 d of age according to published guidelines. An inclusion of 40 mg/kg of Fe in a corn–soybean meal basal diet is a normally added Fe level as determined by a previous study from our laboratory. "2 (d)" represents a single injection interval for every 2 d. The injected Fe concentrations in the saline solution supplemented with either Fe sulphate or 1 of 3 organic Fe sources were 2.08 mg of Fe/ml (10% Fe absorbability solution) and 4.16 mg of Fe/mL (20% Fe absorbability solution) from 22 to 28 d of age, and 2.75 mg of Fe/ml (10% Fe absorbability 115 solution) and 5.50 mg of Fe/ml (20% Fe absorbability solution) from 29 to 35 d of age, and 3.17 mg of Fe/ml (10% Fe absorbability solution) and 6.34 mg of Fe/ml (20% Fe absorbability solution) from 36 to 42 d of age.

Animals and diets

During 1 to 21 d of age, a total of 500 one-d-old Arbor Acres commercial male broilers were fed the same Fe-unsupplemented corn-soybean meal basal diet with all nutrients (except Fe) meeting or exceeding the requirements of starting broilers to enhance their sensitivity to Fe injection. At 22 d of age, 534 birds were

selected according to BW and randomly allotted to 1 of 9 treatments (8 replicate cages of 6 birds per cage) according to above experimental treatments. All injected solutions for all treatments contained the same concentration of methionine or lysine. All birds were fed on the same Fe-unsupplemented corn-soybean meal basal diet with all nutrients, except Fe, meeting or exceeding the requirements for growing.

Birds were housed in electrically heated, thermostatically controlled stainless steel cages coated with plastic and equipped with plastic feeders and waterers. They were handled in accordance with the Arbor Acres Broiler Management Guide for lighting and feeding, and allowed ad libitum access to tap water containing no detectable Fe. Each individual bird was injected with 0.5 mL of either the saline without Fe or with Fe addition through the vein of the wing every other day for 20 d. Feed intake and BW were recorded per cage on d 10 or 20 after injections to calculate ADFI, ADG and FCR during d 1 to 10 or d 10 to 20 after injections.

Sample collections and preparations

On d 10 and 20 after injections, 2 birds from each cage 137 were selected according to the average BW of birds within the cage after fasting for 12 h. Blood samples were taken from each bird via heart puncture with stainless-steel needles equipped with heparinized blood-collection tubes. One part of the blood samples was stored at 4 °C for the analyses of hemoglobin (Hb) concentration and hematocrit (Hct), and the other was centrifuged at 3,000 × g for 10 min at 4 °C to isolate plasma, and then stored at −20 °C until analyses of plasma iron (PI) and total iron binding capacity (TIBC). The birds were subsequently killed by cervical dislocation; and their liver, kidney and heart samples were taken; A subsample was frozen at −20 °C for the analyses of Fe content, and succinate dehydrogenase (SDH), catalase (CAT) or cytochrome c oxidase (COX) activities, and another subsample on d 20 after infections was frozen in liquid N for the assays of CAT, SDH, COX or ferritin heavy chain (FTH1) mRNA and protein levels. The pancreas was also collected and frozen (-20 °C) for Fe concentration determinations. The spleen and right femur marrow samples on d 20 after injections were collected immediately and frozen in liquid nitrogen for the analyses of FTH mRNA and protein levels. The left tibia was excised and frozen in an individual heat-sealed polyethylene bag for Fe content analysis. Tibia bones were ashed in a muffle furnace at 550 °C. Samples of 2 individual chicks from each cage were pooled before analysis, and thus 8 replicate samples were obtained for each treatment.

Sample analyses

Iron concentrations. Iron concentrations in the diets, water, and tissues were determined by inductively coupled plasma emission spectroscope after wet digestion with HNO_3 and $HClO_4$. Validation of the mineral analysis was conducted with the use of bovine liver powder as a standard reference material.

Blood indices. The Hb concentration and Hct in the whole blood were 160 analyzed by an automatic hematology analyzer (ABX Pentra DF 120; HORIBA Medical Inc., Montpellier, France). The PI and TIBC were determined by using the colorimetric method, and transferrin saturation (TS) in the plasma was calculated as PI/TIBC × 100.

Enzyme activities. The liver, heart and kidney samples were homogenized with 10 % (wt./vol.) ice-cold physiologic saline to obtain the homogenates for determination of enzyme activities. The SDH, CAT and COX activities were determined. **mRNA levels.** The primer sequences for Sdh, Cat, Cox, Fth1, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes are shown. The RNA isolation, reverse transcription and real-time qPCR were performed as described previously. The abundances of Cat, Sdh, Cox, and Fth1 mRNA were expressed as ratios of the target gene mRNA to the geometrical mean of β-actin and GAPDH mRNA.

Western blotting

The liver, heart, spleen or femur marrow samples were homogenized in ice-cold RIPA lysis buffer. Then they were centrifuged for 4 min (12,000 × g, 4 °C), and the supernatants were subjected to western-blot analysis. The GAPDH protein was used to normalize the expression levels of SDH, CAT, COX or FTH protein.

Statistical analyses

To test the effect of the injected Fe, a single degree of freedom contrast was used to compare all injected Fe treatments with the control. Data excluding the control were further analyzed by two-way ANOVA with a model that included the main effects of the injected Fe source, injected Fe concentration and their interaction using the general linear model procedure of SAS. The replicate cage served as the experimental unit. Differences among the means were 183 tested by the LSD test. $P \leqslant 0.05$ was considered to be statistically significant.

III. Discussion

The results from the present study indicated that based on the liver Fe concentration on d 10 or 20 after Fe injections, and PI and femur marrow FTH1 protein expression level on d 20 after Fe injections, the intravenously injected organic Fe source with extremely strong chelation strength had the lowest Fe utilization in the target tissues of broilers, which has supported our hypothesis.

Our results provided scientific experimental bases for developing and applying the organic Fe sources with appropriate chelation strengths and high metabolic utilization of Fe in broiler production. The intrinsically labeled radioisotope method is a good way to verify mineral metabolism and utilization in animals. In the present study, we did not adopt this method because none of the commercial organic Fe products used had been manufactured with intrinsic radiotracers or stable isotopes of Fe. The intravenous injection method was considered an effective method to detect differences in metabolic utilization of minerals in the sensitive target tissues of animals. Therefore, the intravenous injection technique was used in the current study to detect differences in the metabolic utilization of Fe from different Fe sources in the sensitive target tissues of broilers. As an evaluation marker of utilization, growth observation is generally unresponsive for many trace elements. In the present study, injected Fe source did not influence growth performance of birds, which is in line with our previous studies, indicating that growth performance might be affected by factors other than Fe source, and was not sensitive for assessment of the metabolic utilization of Fe sources for broilers.Hematologic indices are commonly used to assess iron status for chicks. In the present study, Hb concentration on d 20 after Fe injections and Hct on d 10 or 20 after Fe injections increased as the injected Fe dosage increased. Blood Hb concentrationand Hct have been considered as responsive criteria to assess the bioavailabilities of Fe reported that blood Hb concentration was a sensitive index in reflecting 297 differences in bioavailability among different Fe sources. The disparity might be mainly due to the different diets used in the above 2 studies. The PI represents the Fe concentration that binds to transferrin. The results from the present study indicated that PI, but not Hb and Hct, was a sensitive indicator to detect the differences in metabolic utilisation of injected Fe among Fe sources. The Fe from injected Fe-ProtES was less utilizable for PI accumulation in broilers on d 20 after injection than that from injected FeSO4·7H2O. The different methods of Fe administration (present intravenously injection vs. dietary supplementation. might partially explain the inconsistency. Previous studies in broilers demonstrated that Fe concentrations in the liver and kidney, especially in the liver, increased as dietary Fe concentration increased. Our present study indicated that liver and kidney Fe concentrations increased as the injected Fe dosage increased, which was similar to the previous findings, suggesting that the injected Fe from different Fe sources could be mobilized and deposited in the liver and kidney of chickens. Target tissue accumulations of trace minerals have been considered to be sensitive criteria for assessment of their bioavailabilities. In the present study, liver and kidney Fe concentrations on d 20 after Fe injections were sensitive enough to detect the differences in the tissue utilization of injected Fe among Fe sources. Based on these sensitive criteria, the Fe from the injected Fe-ProES was less utilizable for liver and kidney tissues of broilers than that from the injected Fe-MetW, Fe-ProtM or FeSO4·7H2O. These results are in agreement with those of previous, which showed that the injected 320 organic Mn or Zn source with strong chelation strength was the least favorable for tissue Mn or Zn utilization by broilers.

Iron is vital for the functions of numerous iron-containing enzymes, such as CAT, SDH and COX. Research with broilers and pigs has demonstrated that the CAT activities in the liver increased as dietary Fe levels increased. Similarly, the results from the present study indicate that CAT activity in the liver on d 10 after injections increased as the injected Fe dosages increased, suggesting that injected Fe can be utilized in the synthesis of CAT in the liver of chickens. The same trends were observed in a study on broilers. The results from the current study showed that SDH activity in the liver on d 10 after injections reduced as the injected Fe dosages increased, implying that high Fe addition might downregulate the synthesis of SDH in the liver. Our previous3 results indicated that the COX activity in heart increased quadratically as dietary Fe level increased, and it was sufficiently sensitive to evaluate Fe status and Fe requirements for broilers fed a maize-soybean-meal diet from 22 to 42 d of age. It observed that COX activity in the brain of rats increased as supplemental Fe increased. In the present study, COX activity in the heart on d 10 or 20 after injections increased as the injected Fe dosages increased, which is consistent with the previous results. Additionally, in our present study, no differences were found in the CAT, SDH or COX activities in the liver, heart and kidney among Fe sources, indicating that these enzyme activities in the tissues lack enough sensitivity to detect the differences in tissue utilization of injected Fe from different Fe sources in broilers.

The gene expression of Fe-containing enzymes might be another 342 type of new and more sensitive biomarker to reflect the iron status in the body of chickens.

Ferritin is a ubiquitous intracellular Fe storage protein. Although the genomes of many species contain multiple copies of heavy-chain and light-chain sequences, the chicken genome contains only a single copy of the heavy-chain gene. Ferritin has been found in many tissues, and is deposited mainly in the spleen, liver, and bone marrow. The expression of ferritin is tightly controlled by the intracellular Fe concentration. When the Fe level

is low, the expression of ferritin is suppressed to avoid intracellular Fe sequestration. The opposite scenario takes place as Fe is abundant. Iron modulates ferritin synthesis post-transcriptionally in animals. It reported that dietary supplemental Fe increased the protein expression of FTH1 in the brain of rats. The present study demonstrated that FTH1 protein expression in the liver and spleen increased as the injected Fe dosages increased, which is in line with the results. To our knowledge, no information is available regarding the effect of supplemental Fe as different Fe sources on the FTH1 protein expression in the tissues of chickens. In the current study, FTH1 protein expression levels in 388 the spleen of broilers on d 20 after injections were sensitive enough to detect differences in the tissue utilization of injected Fe in broilers among Fe sources. Based on the criterion, intravenously injected Fe from Fe-ProtES was the least utilizable Fe source. This might be due to its extremely strong chelation strength of the bonds between Fe and ligands, which retarded Fe from the organic Fe source being mobilized for metabolic utilization in the target tissues of broilers. The results from the present study and our previous studies suggest that more organic Fe from Fe-ProtES could better resist interference from the low pH in the stomach and complex factors in the gut and directly reach the intestinal brush border, where it is hydrolyzed and absorbed as an ion, resulting in higher Fe bioavailability. The results from the present study also indicate that there are differences not only in the absorption of Fe in the small intestine, but also in the metabolic utilization of Fe from organic Fe sources with different chelation strengths in the target tissues of broilers. Obviously and surely, in practice with dietary supplemental Fe, we should choose organic Fe with strong chelation strength because it has the highest bioavailability reflecting both the Fe absorption in the gut and metabolic Fe utilization in the target tissues of broilers. However, further studies will be needed to address the relative bioavailability, absorption and utilization of Fe from the most strongly chelated EDTA Na-Fe in broilers.

IV. Conclusions

In this paper study indicated that liver and kidney Fe concentrations, and liver Cox mRNA levels and spleen FTH1 protein expression levels were sensitive enough for detecting differences in tissue utilization of injected Fe from different Fe sources in broilers.

Based on the above biomarkers, intravenously injected Fe from the organic Fe source with extremely strong chelation strength was the least utilizable Fe source and functioned in the sensitive target tissue less effectively than Fe from Fe sulfate or the other 2 organic Fe sources with weak or moderate chelation strength. These findings might provide a further insight into the utilization mechanism of Fe in the target tissues of chickens.

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